Patent Application
Docket No. UF-155CD1
Serial No. 09/070,844

THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Ousama Zaghmout
Art Unit	:	1649
Applicants(s)	:	Robert R. Schmidt, Philip Miller
Serial No.	:	09/070,844
Filed	:	May 1, 1998
For	:	Novel Polypeptides and Polynucleotides Relating to the α- and β-Subunits of Glutamate Dehydrogenases and Methods of Use

Assistant Commissioner for Patents
Washington, D.C. 20231

SECOND DECLARATION OF PHILIP MILLER, Ph.D.

Sir:

I, Philip Miller, Ph.D. hereby declare and say as follows:

THAT, my qualifications have been set forth in my July 30, 1998 declaration prepared for and filed in grandparent application Serial No. 08/541,033;

THAT, I have studied the application Serial No. 09/070,844 and all office actions and responses filed therein, including the Office Action mailed September 14, 1999 in that application; and being thus duly qualified further declare as follows:

1. Attached hereto as Exhibit A is a summary of experimental results demonstrating that the assimilatory *E. coli* GDH is capable of enhancing nitrogen uptake and growth in plants (*Arabidopsis*), thereby confirming the truth of our teachings in the application that polynucleotides encoding proteins having GDH enzymatic activity (other than *Chlorella* GDH) can be used to modulate nitrogen assimilation in plants. Those of ordinary skill in the art have reported similar results in a wide variety of plant species including, for example, tobacco, corn, wheat, and soybeans, clearly proving the broad applicability of our teachings. (See, for example, U.S. Patent No. 5,998,700 issued to Lightfoot *et al.*).

2. As explained in my previous declaration, one of ordinary skill in the art can routinely generate fragments of any known GDH polynucleotide using, for example, *Bal*31 exonuclease. It is well known in the art that a vast number of polynucleotides encoding functional proteins can be truncated into fragments which encode shorter polypeptides retaining the original activity. In view of our teachings that fragments of GDH polynucleotides can be used to encode polypeptides exhibiting GDH activity, and in view of our teachings of assays to verify such activity, one of ordinary skill in the art would truly be surprised if such fragments could not be generated. Further, because of the fact that the techniques necessary for such genetic manipulation have been routine for more than fifteen years, and assays for the desired activity are explicitly taught in the application, useful fragments of any full-length GDH polynucleotide can be routinely generated by anyone of ordinary skill in the art.

3. The assertion that Brears *et al.* (WO 95/09911 A) discloses our invention is simply incorrect. One of ordinary skill in the art, reading Brears in the absence of our teachings, would not be able to practice the claimed invention. Our application teaches that GDHs exist that have different kinetic properties (aminating versus deaminating), and that by knowing these properties one skilled in the art can modify nitrogen metabolism in crop plants to increase yields, biomass, and nutritional properties. In contrast, Brears provides no teaching on which GDH to use under which circumstances, no teaching concerning how by knowing the properties of GDH one can utilize GDH to favor nitrogen compound formation or nitrogen compound degradation, and thus no teaching to one of ordinary skill to modulate nitrogen metabolism in plants. The cited reference simply lists GDH in a laundry list of enzymes without providing any detailed description of how to use GDH, and therefore does not enable the ordinarily skilled artisan to practice the invention. In addition to failing to provide teachings regarding how to use GDH for modulating nitrogen metabolism, Brears *et al.* provide no evidence that would give the ordinarily skilled artisan any reasonable expectation that nitrogen metabolism could be modulated by use of GDH. Such a teaching is required because those skilled in the art did not expect it could be done. No evidence is presented in the cited reference to demonstrate that such modulation is even possible. Brears *et al.* states, "Since both of these enzymes (GDH and AS) have a high Km, the roles of these alternate nitrogen

assimilation pathways under normal growth conditions remain unclear." Not only does this statement fail to provide any information on how to use GDH to achieve increased plant growth and yield, but it argues against the significance of GDII for playing a role in carbon or nitrogen metabolism. Significantly, in a July 1995 publication by one of the Brears *et al.* co- applicants, it is argued that the role of GDH is not known, and that it may only degrade glutamate. In contrast to the teachings of the subject application, where it is disclosed not only that multiple forms of GDH exist, but also which forms assimilate nitrogen and which deaminate glutamate, how to determine which forms do which, and further how the different forms can be used to achieve increased biomass, yield, nitrogen metabolism, etc., the Brears *et al.* co-applicants state as follows:

> "Furthermore, the GDH enzyme has a high Km for ammonia, a characteristic that argues against an assimilatory role and suggests a catabolic role for this enzyme. Another possible biological role for GDH is in ammonia detoxification process. This role has been suggested based on the observation that this enzyme is induced by high levels of ammonia. Mitochondrial GDH has been proposed to be involved in the assimilation of high concentrations of photo respiratory ammonia released in the mitochondria. However, the isolation of photo respiratory mutants defective in chloroplastic GS2 and Fd; GOGAT argues against a major role for GDH in this process.... In conclusion, despite the bulk of information on the biochemistry of plant GDH, the actual function of this enzyme in higher plants remains elusive." (H. M. Lamb, *et al.* (G. Coruzzi), *Plant Cell* 7:887-898).

4. Further, in May 1987, Brears *et al.* co-applicant G. Coruzzi persists with the statements that she and others in the field do not know the relevance or utility of GDH in plants: "In summary, despite all the data accumulated on [*sic*, in] the last several years on the biochemistry of the GDH enzymes, the actual *in vivo* role of this enzyme in plant nitrogen metabolism remains unresolved." I. C. Oliveira *et al. Plant Physiology and Biochemistry*, 35:185-198 (1997). Accordingly, it is clear that Brears *et al.*, and others of ordinary skill in the art in the absence of the teachings of this application have no understanding of the role or utility of GDH, or of the utility of over-expressing this nitrogen enzyme to enhance plant growth or nutrition.

5. The deficiencies of Brears *et al.* are further illustrated by the comments of the reviewers

of the Schmidt grant proposal, which is attached as Exhibit C to the Declaration of Robert Schmidt, Ph.D. dated July 30, 1998 in related application Serial No. 08/541,033. As a review of that exhibit will clearly indicate, the disclosure of the grant proposal is in much more detail than what is provided by the Brears *et al.* publication, and therefore provides more detailed, explicit guidance to one of ordinary skill in the art. Despite this more complete disclosure, the assertions of Dr. Schmidt were still doubted by the peers who reviewed the grant proposal. In their comments one finds evidence that those of skill in the art doubted that GDH could have the nitrogen metabolism modulating effects that we have taught in this application. This leads to the inescapable conclusion that Brears *et al.*, who didn't provide any detailed instructions regarding use of GDH in plants nor any data demonstrating the effect of GDH on nitrogen metabolism in plant cells does not enable one of skill in the art to practice the claimed invention, and therefore does not anticipate the claimed invention.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information in belief are believed to be true; and further that these statements were made with the knowledge that willful false statements in the like so made are punishable by fine or imprisonment, or both, under §1001 of title 18 of the U.S.C. and that such willful false statements made jeopardize the validity of the application or of any patent issuing thereon, or mutant of the claimed sequence.

Further declarant sayeth naught.

Philip Miller, Ph.D.

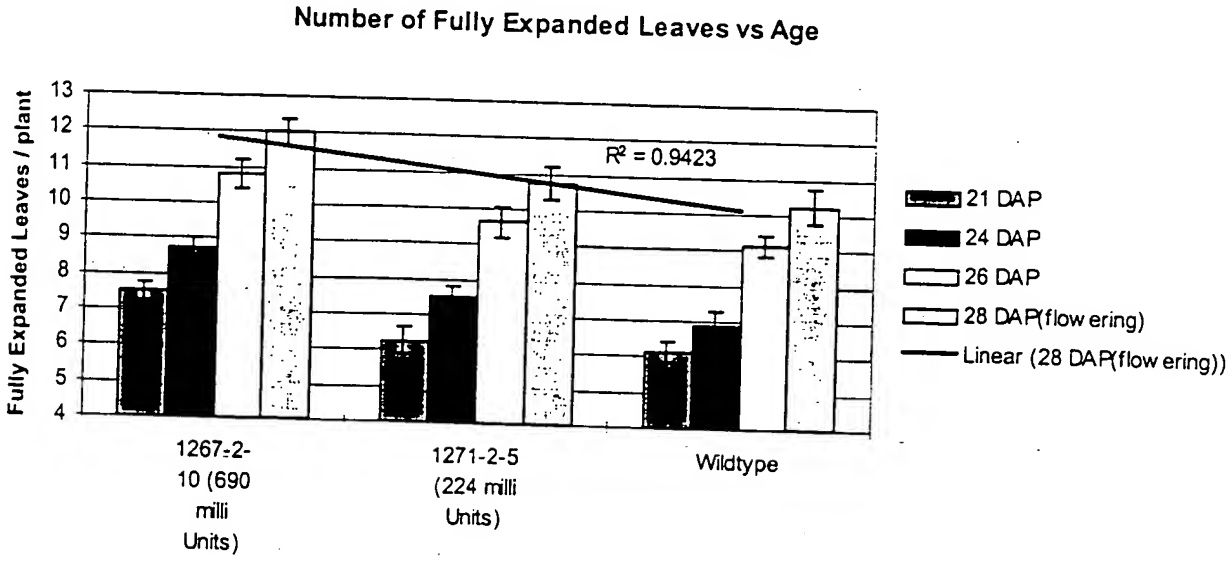
3/10/00
Date

Evaluation of *Arabidopsis* Events Expressing *E.coli* GDH -- P. Miller

We have three different transgenic *Arabidopsis* constructs in the area of primary nitrogen metabolism with the hypothesis that the transgenes will enhance nitrogen assimilation. To date we have shown that overexpression of an assimilatory GDH gene (*Chlorella* alpha GDH) in *Arabidopsis* can enhance nitrogen assimilation by 24%, as measured by total vegetative N levels, where a deaminating version of GDH (*Chlorella* beta GDH) causes a decrease in total nitrogen levels. To confirm this affect, *Arabidopsis* plants were transformed with a different assimilatory GDH gene (*E.coli* GDH) with the hypothesis that all assimilatory GDH genes can enhance nitrogen assimilation. To initiate these experiments, we have chosen two homozygous positive events expressing different levels of the *E.coli* GDH protein (e35s-*E.coli* GDH). The objective of the experiment was to try and understand how *E.coli* GDH expression affects biomass and leaf total carbon/nitrogen levels.

In the most recent interim, the biomass experiment was completed on two *E.coli* GDH events. The data of that analysis will be reported here. (Figure TR1).

Figure TR1:



These results show that *Arabidopsis* plants expressing *E.coli* GDH have significantly more leaves throughout their development as compared to wild type plants (control). Secondly, the trend for increased leaf number is correlated with *E.coli* GDH expression level. The increases in biomass and growth are similar to those demonstrated with the assimilatory *Chlorella* GDH and demonstrates that other assimilatory GDH's provide utility in modifying nitrogen metabolism which

leads to protein increases in seeds and plants, increased kernel density in grain crops, and yield as demonstrated with the assimilatory *Chlorella* GDH.
Measures of total nitrogen demonstrated that, as previously reported for *Arabidopsis* plants over-expressing Chlorella alpha-GDH, plants which expressed the *E. coli* GDH assimilatory form showed a significant increase in plant nitrogen content. See below

Arabidopsis Plant event #	umol Nitrogen/gm fw	standard deviation
Alpha-GDH Chlorella	20.3	0.012
E. coli event 1267	26.9	0.012
E. coli 1271	39.2	0.03
Wild type Arabidopsis	12.9	0.012

This provides demonstration that other assimilatory forms of GDH from alternative organisms do enhance nitrogen assimilation of plants. Alternative assimilatory forms that have been expressed in plants also include an assimilatory GDH form Synechosystis 6803.
The *E. coli* GDH has also been demonstrated to express in monocot species with wheat providing the demonstration.